ATP-Induced Phosphorylation of the Sarcoplasmic Reticulum Ca^{2+} ATPase: Molecular Interpretation of Infrared Difference Spectra

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ABSTRACT Time-resolved infrared difference spectra of the ATP-induced phosphorylation of the sarcoplasmic reticulum Ca^{2+}-ATPase have been recorded in H_2O and ^2H_2O at pH 7.0 and 1°C. The reaction was induced by ATP release from P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate (caged ATP) and from [γ-$^{18}O_3$]caged ATP. A band at 1546 cm^{-1}, not observed with the deuterated enzyme, can be assigned to the amide II mode of the protein backbone and indicates that a conformational change associated with ATPase phosphorylation takes place after ATP binding. This is also indicated between 1700 and 1610 cm^{-1}, where bandshifts of up to 10 cm^{-1} observed upon protein deuteration suggest that amide I modes of the protein backbone dominate the difference spectrum. From the band positions it is deduced that α-helical, β-sheet, and probably β-turn structures are affected in the phosphorylation reaction. Model spectra of acetyl phosphate, acetate, ATP, and ADP suggest the tentative assignment of some of the bands of the phosphorylation spectrum to the molecular groups of ATP and Asp^{351}, which participate directly in the phosphate transfer reaction: a positive band at 1719 cm^{-1} to the C=O group of aspartyl phosphate, a negative band at 1239 cm^{-1} to the $\nu_{as}(PO_2^-)$ modes of the bound ATP molecule, and a positive band at 1131 cm^{-1} to the $\nu_{as}(PO_3^{2-})$ mode of the phosphoenzyme phosphate group, the latter assignment being supported by the band's sensitivity toward isotopic substitution in the γ-phosphate of ATP. Band positions and shapes of these bands indicate that the α- and/or β-phosphate(s) of the bound ATP molecule become partly dehydrated when ATP binds to the ATPase, that the phosphoenzyme phosphate group is unprotonated at pH 7.0, and that the C=O group of aspartyl phosphate does not interact with bulk water. The Ca^{2+} binding sites seem to be largely undisturbed by the phosphorylation reaction, and a functional role of the side chains of Asn, Gln, and Arg residues was not detected.

INTRODUCTION

The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase couples active Ca^{2+} transport to the hydrolysis of ATP. For reviews see Andersen (1989, 1995), Inesi et al. (1995), and Mintz and Guillain (1995). In an essential step, Asp^{351} of the Ca^{2+}-loaded form of the ATPase reacts with the γ-phosphate of ATP to form the ADP-sensitive phosphoenzyme Ca_2E_1-P. This reaction prevents access from the cytoplasm to the Ca^{2+}-binding sites; the bound Ca^{2+} ions are then occluded in the protein (Mintz and Guillain, 1995, 1997; Vilsen, 1995). Although mutagenesis studies have identified crucial residues for this reaction (Andersen, 1995), the molecular mechanism remains obscure in the absence of a structure at atomic resolution.

Structural changes of the Ca^{2+}-ATPase during its reaction cycle can be investigated by reaction-induced infrared difference spectroscopy (Barth et al., 1990, 1991, 1994, 1996; Buchet et al., 1991a, 1992; Georg et al., 1994; Troullier et al., 1996). The method uses the release of effector molecules from biologically "silent" photolabile derivatives, i.e., caged compounds (McCray and Trentham, 1989; Corrie and Trentham, 1993), to generate high-quality infrared difference spectra. The reaction products and infrared difference spectra of caged ATP photolysis and of side reactions have been characterized recently (Barth et al., 1995, 1997a).

Using time-resolved Fourier transform infrared (FTIR) spectroscopy, we have obtained spectra of the ATP-induced phosphorylation of the ATPase (Barth et al., 1996). The present work studies in detail the structural changes associated with this reaction, using H_2O and ^2H_2O as the solvent and isotopically labeled caged ATP ([γ-$^{18}O_3$] caged ATP). Conformational changes of the protein, and the molecular groups directly participating in the phosphorylation reaction are discussed.

MATERIALS AND METHODS

Sample preparation

Samples for time-resolved infrared spectroscopy were prepared as described previously (Barth et al., 1994, 1996) by removal of free water from an SR suspension in a stream of nitrogen. Samples were immediately rehydrated with H_2O or ^2H_2O with or without 20% Me_2SO. This method resulted in active ATPase samples (Barth et al., 1991). Approximate concentrations for the two types of sample used are given in Table 1.

FTIR measurements

Time-resolved FTIR spectra of the $Ca_2E_1 \cdot ATP \rightarrow Ca_2E_1$-P reaction were recorded at 1°C with a modified Bruker IFS 66 spectrometer as described previously (Barth et al., 1996). From these spectra, difference spectra for the phosphorylation reaction were calculated by subtracting a spectrum of $Ca_2E_1 \cdot ATP$ from a spectrum of Ca_2E_1-P. The spectrum of $Ca_2E_1 \cdot ATP$ for type I samples was averaged in the time interval from 71 ms to 644 ms after triggering of caged ATP photolysis, for type II samples between 71

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TABLE 1 Approximate concentrations of ATPase and other constituents in the ATPase samples

Sample name	ATPase (mM)	Buffer	pH	K^+ (mM)	Ca^{2+} (mM)	Glutathione (mM)	Caged ATP (mM)	A23187 (mg/ml)	Adenylate kinase (mg/ml)	Me_2SO
Type I sample	0.7	300 mM MOPS	7.0	330	10	20	20	0.5	2	—
Type II sample	0.7	300 mM Imidazole	7.0	—	1	20	20	0.5	2	20%

Concentrations are based on a 1-μl sample volume and 4.5 nmol active ATPase·mg protein (Nakamura et al., 1982; Shigekawa et al., 1983; Fernandez-Belda et al., 1984; Inesi and De Meis, 1985; Andersen et al., 1985; Inesi, 1987). The volume of the samples used varied between 0.6 and 1.2 μl. MOPS, 3-(*N*-morpholino)propanesulfonic acid.

and 385 ms. The spectrum of Ca_2E_1-P was obtained between 3.2 and 11.0 s, except for the ^2H_2O type II samples, where phosphorylation is slowest (Barth et al., 1996), and the spectrum was obtained between 18.8 and 11.0 s. Spectra of type II samples were corrected as described for E_2-P already formed (Barth et al., 1996).

A normalization of spectra to an identical protein concentration is not necessary to generate the difference spectra, because they are obtained directly from the time-resolved absorbance changes of individual samples. However, to prevent the possible predominance of individual samples with high protein content in the averaged difference spectra and for a better comparison of samples in H_2O and ^2H_2O, the difference spectra were normalized to an identical protein concentration before averaging. For this, difference spectra were multiplied with the same factor that was needed to normalize the respective absorbance spectra measured in H_2O to an amide II absorbance of 0.26 (difference in absorbance between 1546 and 1492 cm^{-1}) and the spectra measured in ^2H_2O to an amide I absorbance of 0.47 (difference in absorbance between 1706 and 1648 cm^{-1}). The amide II band was chosen for the normalization of H_2O samples because the amide II band is less sensitive to errors in water subtraction. The link between the normalization of H_2O and ^2H_2O samples was done via the ratio of the amide II to amide I absorption of a completely dried ATPase film, assuming that the amide I intensity is only marginally affected by protein deuteration and that the ratio of amide II to amide I absorption is unaffected by drying. The latter seems to be justified by ATR experiments showing that thick films of ATPase are unordered and that the ratio of amide II to amide I absorption is approximately the same for conditions preserving the native ATPase structure and for those that denature the protein (Buchet et al., 1991b). However, the assumption that the amide I intensity is nearly unaffected by protein deuteration may only approximately be valid. Studies of peptides show that whereas the extinction coefficient for β- and random coil structures is nearly the same in H_2O and ^2H_2O, this is not the case for α-helical structures (Chirgadze et al., 1973; Chirgadze and Brazhnikov, 1974; Venyaminov and Kalnin, 1990b). Their extinction coefficient is 1.5 times larger in H_2O. For proteins, however, $H/^2H$ exchange studies of several proteins, including H^+,K^+-ATPase, showed only minor effects of protein deuteration on the intensity of the amide I band (Goormaghtigh et al., 1994; De Jongh et al., 1997a,b; Raussens et al., 1997).

Absorbance spectra of the model compounds acetyl phosphate (pH 6.7), Mg^{2+} acetate (pH 8.5), ATP, and ADP (both pH 7.9) were recorded with the same instrument at 20°C and the same set of CaF_2 windows (one of which had a trough to receive the sample volume). Small variations in the path length between different samples were corrected for by normalizing each spectrum to a standard H_2O spectrum, using the water band at 2130 cm^{-1}. Sample concentrations were 200–500 mM, and the spectra were normalized to 500 mM sample concentration.

The model difference spectrum for the phosphorylation reaction was obtained by calculating the absorbance of acetyl phosphate and ADP minus the absorbance of acetate and ATP.

RESULTS

The difference spectrum of ATPase phosphorylation

Fig. 1 shows the phosphorylation spectrum for type I and type II samples. These differ in their buffer composition, with the effect that the decomposition of Ca_2E_1-P is inhibited in type I samples but not in type II samples (Barth et al., 1996). The two types of sample show very similar phosphorylation spectra (compare *solid lines* in Fig. 1, *A* and *B*), indicating that the same enzyme state is adopted by the two sample types. Phosphorylation rates of type I and type II samples agree approximately for experiments performed in H_2O, but are 20–30% lower for type II samples in ^2H_2O (Barth et al., 1996). Negative bands are characteristic for the initial enzyme state $Ca_2E_1 \cdot ATP$, positive bands for Ca_2E_1-P. In general, the changes in infrared absorbance upon ATPase phosphorylation are very small, less than 0.2% of the total protein absorbance (Barth et al., 1996). To help assign bands to molecular groups, absorbance spectra of acetyl phosphate, acetate, ATP, and ADP were recorded and various experimental conditions were employed, including deuteration of the ATPase and the use of isotopically labeled caged ATP.

The effects of deuteration

Deuteration of proteins helps distinguish between bands caused by the amide modes of the polypeptide backbone from bands due to the amino acid side chains. For the amide I modes (1700–1610 cm^{-1}), band shifts up to 15 cm^{-1} upon deuteration are expected (Susi et al., 1967; Byler and

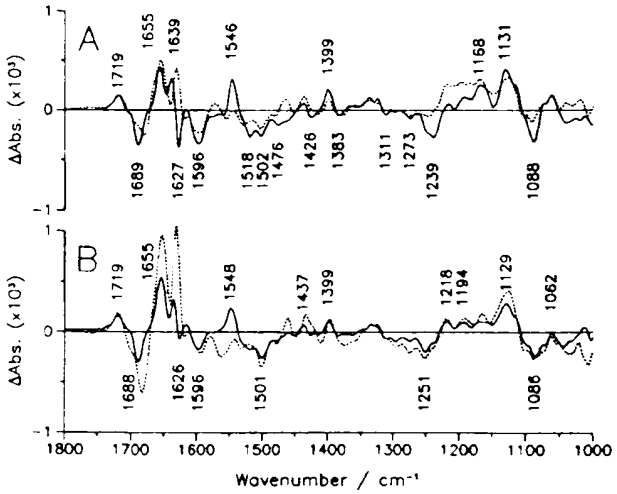

FIGURE 1 Phosphorylation spectrum in H_2O (——, pH 7.0) and ^2H_2O (---, p^2H 7.0), 1°C. (*A*) Type I samples. (*B*) Type II samples.

Susi, 1986; Haris and Chapman, 1992; Arrondo et al., 1993; Jackson and Mantsch, 1995). Amino acid side chains absorbing in that region, i.e., those from Asn, Gln, Lys, Arg, show larger shifts (Chirgadze et al., 1975; Venyaminov and Kalnin, 1990a). The amide II band shifts from ~1550 cm^{-1} to 1450 cm^{-1} (Byler and Susi, 1986; Haris and Chapman, 1992).

In the amide I and amide II regions, several differences between the phosphorylation spectra obtained in H_2O and ^2H_2O are observed (see Fig. 1 *A*). The band at 1689 cm^{-1} shifts to 1680 cm^{-1}, the band at 1655 cm^{-1} shifts slightly to 1654 cm^{-1}, the band at 1639 cm^{-1} shifts to 1630 cm^{-1}, and the band at 1627 cm^{-1} to 1618 cm^{-1}, where the adjacent positive band is superimposed on it. These shifts of up to 10 cm^{-1} are indicative of amide I bands. Because no large shifts are observed, as expected for the amino acid side chains absorbing in the amide I region (Asn, Gln, Lys, and Arg), we conclude that the 1700–1610 cm^{-1} region of the phosphorylation spectrum is dominated by changes in the amide I absorbance of the polypeptide backbone. The extent of the conformational change is very small (Barth et al., 1996), and it is likely that the absorbance changes reflect modifications within existing secondary structure elements. The band positions involved are characteristic for β-sheet (1689, 1639, and 1627 cm^{-1}), turn (1689 cm^{-1}), and α-helical structures (1655 cm^{-1}). The band pair at 1639/1627 cm^{-1} may indicate a shift of a β-sheet band. It is expected to be accompanied by a band near 1690 cm^{-1} with weaker intensity (Susi et al., 1967; Chirgadze et al., 1973; Venyaminov and Kalnin, 1990b; Arrondo et al., 1993; Pribic et al., 1993), where indeed a negative band is observed. However, this band is relatively large in intensity compared to the band pair at 1639/1627 cm^{-1}, which suggests that this band may not exclusively originate from β-sheet structures, and that turn structures may also contribute.

A conformational change is also indicated by the 1546 cm^{-1} band, which can be assigned to the amide II mode of the backbone because it is no longer present for the deuterated enzyme. Instead, for the deuterated enzyme a band is observed at 1464 cm^{-1}, which may represent the amide II′ mode.

The side chains of Asn, Gln, and Arg have relatively high extinction coefficients in the 1610–1700 cm^{-1} region, and are expected to be observable in the infrared difference spectrum if they experience a change in interaction with the environment. However, no large band shifts were detected upon deuteration, which are expected for these side chains if they are accessible to deuteration. Asn, Gln, and Arg residues on the protein surface of the ATP-binding and phosphorylation site are expected to be accessible to deuteration. Thus there is no evidence from the spectra for a change of side-chain interactions of those residues upon ATPase phosphorylation.

Spectra of model compounds

Phosphorylation of the Ca^{2+}-ATPase converts ATP to ADP and transfers its terminal phosphate to Asp^{351}. In the absence of specific interactions, the carboxyl group of Asp^{351} should be deprotonated at neutral pH. Fig. 2, *A* and *B*, shows absorbance spectra of model compounds in H_2O for the phosphorylation reaction. In Fig. 2 *A* the bands of ATP and ADP above 1300 cm^{-1} can be assigned to the adenine and ribose moiety of the nucleotides (Shimanouchi et al., 1964). As expected, vibrations of that part of the molecule are not affected by the removal of the terminal phosphate group of ATP (the small difference in intensity at 1654 cm^{-1} is due to the difficulties of water subtraction in that spectral region). Below 1300 cm^{-1}, differences are observed between the ATP and the ADP spectrum that can be attributed to the phosphate groups. At 1231 cm^{-1} (ATP) and 1211 cm^{-1} (ADP), the ν_{as} vibration(s) of the PO_2^- group(s) absorb(s) (Takeuchi et al., 1988). The $\nu_s(PO_2^-)$ and $\nu_{as}(PO_3^{2-})$ vibrations contribute to the absorbance near 1110 cm^{-1} (Takeuchi et al., 1988).

In Fig. 2 *B* the absorbance spectra of acetyl phosphate and Mg^{2+} acetate are shown. The bands are assigned as follows based on acetate, acetate ester (Colthup et al., 1975), or alkyl phosphate spectra (Shimanouchi et al., 1964): acetyl phosphate: 1718 cm^{-1} ν(C=O), 1376 cm^{-1} $\delta_s(CH_3)$, 1273 cm^{-1} ν(C-O), 1132 cm^{-1} $\nu_{as}(PO_3^{2-})$; acetate: 1553 cm^{-1} $\nu_{as}(COO^-)$, 1417 cm^{-1} $\nu_s(COO^-)$. Protonation of acetyl phosphate affects the electron density distribution of the phosphate and the carboxyl moieties and thus shifts the respective bands. They are observed (data not shown) at 1738 cm^{-1} for ν(C=O), 1237 cm^{-1} for $\nu_{as}(PO_2^-)$, and 1097 cm^{-1} for $\nu_s(PO_2^-)$.

Fig. 2 *C* shows a model difference spectrum for the phosphorylation reaction based on the model compound

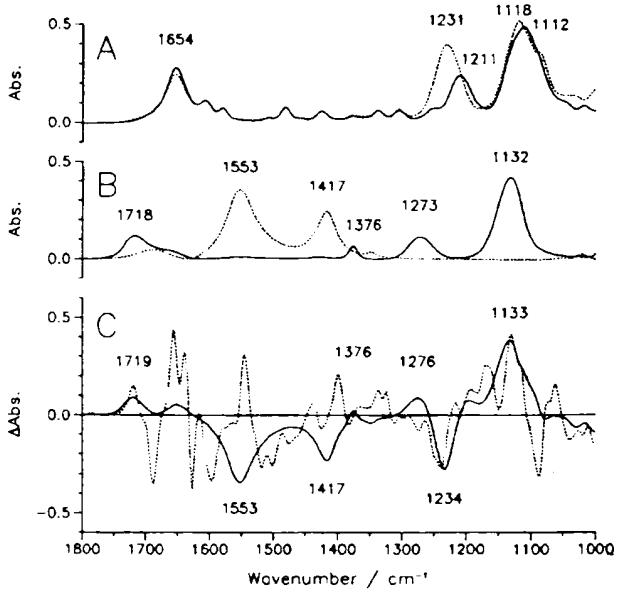

FIGURE 2 Model compound spectra in H_2O. (*A*) ——, ADP, pH 7.9; ----, ATP, pH 7.9. (*B*) ——, Acetyl phosphate, pH 6.7; ----, Mg^{2+} acetate pH 8.5. (*C*) ——, Model difference spectrum, i.e., absorbance of acetyl phosphate and ADP minus absorbance of acetate and ATP; ----, phosphorylation spectrum of type I samples in H_2O multiplied by 1000.

absorbance spectra (*solid line*). The model difference spectrum was calculated as the absorbance of acetyl phosphate and ADP minus the absorbance of acetate and ATP, and thus reflects changes in the infrared spectrum that are expected for the molecular groups that are directly involved in the phosphorylation reaction, i.e., Asp^{351} and ATP:

$$AMP{-}(PO_2^-){-}O{-}(PO_3^{2-}) + Asp^{351}{-}COO^- \rightarrow AMP{-}(PO_3^{2-}) + Asp^{351}{-}(C{=}O){-}O{-}(PO_3^{2-})$$

Negative bands in the model difference spectrum indicate the disappearance of the acetate COO^- group (1553 and 1417 cm^{-1}) and of one PO_2^- group of ATP (1234 cm^{-1}). Positive bands show the appearance of the C=O group (1719 cm^{-1}), of the C-O group (1276 cm^{-1}), and of the additional PO_3^{2-} group (1133 cm^{-1}) of acetyl phosphate. The small band at 1376 cm^{-1} was assigned to the CH_3 group of acetyl phosphate and is not expected to appear in the phosphorylation spectrum, because there is no CH_3 group in Asp^{351}. The small band near 1650 cm^{-1} is due to the difficulties of accurate water subtraction in that spectral region. It is not present in the respective difference spectrum with the solvent ^2H_2O, which does not absorb in that region (data not shown).

Comparison of the model difference spectrum with the phosphorylation spectrum

Fig. 2 *C* also shows the ATPase phosphorylation spectrum (*dotted line*, multiplied by a factor of 1000). Some of the bands of the phosphorylation spectrum also appear in the model difference spectrum. On this basis, we tentatively assign the 1719 cm^{-1} phosphorylation band to the C=O group of aspartyl phosphate (Barth et al., 1994), the band near 1234 cm^{-1} to the $\nu_{as}(PO_2^-)$ modes of the α- and β-phosphate groups of ATP bound to the ATPase, and the band near 1133 cm^{-1} to the PO_3^{2-} groups of ADP and aspartyl phosphate. The negative band at 1088 cm^{-1} is most likely caused by a $\nu_s(PO_2^-)$ band of ATP, which is seen as a shoulder at 1088 cm^{-1} in solution (see Fig. 2 *A*) (Takeuchi et al., 1988). The overall similarity of the two spectra below 1250 cm^{-1} suggests that this region of the phosphorylation spectrum may be dominated by changes in phosphate absorption. However, there are two relatively strong bands expected in this region that are not associated with ATPase phosphorylation, a positive band at 1257 cm^{-1} and a negative band at 1149 cm^{-1}. They were attributed to the side reaction of thiol reagents with the photolysis by-products (Barth et al., 1996).

The expected negative bands due to the disappearance of the carboxylate group at 1553 and 1417 cm^{-1} are not obvious in the phosphorylation spectrum. However, there is a small negative band at 1426 cm^{-1} (Fig. 1 *A*) that is present at a similar position in type I and type II samples in H_2O and ^2H_2O (Fig. 1). The intensity of this negative band may be larger than seen in the spectra because of overlap with the positive band at 1399 cm^{-1}. A negative band at 1553 cm^{-1} is not observed in the phosphorylation spectra in H_2O. In ^2H_2O, however, the disappearance of the positive band at 1546 cm^{-1} reveals an underlying negative band at 1555 cm^{-1}. Other candidates for the two Asp^{351} carboxylate bands are the bands at 1596 and 1383 cm^{-1}, if special interactions with the environment are assumed (Deacon and Phillips, 1980; Tackett, 1989).

Several bands of the phosphorylation spectrum in H_2O do not have related bands in the model difference spectrum. Some of these are likely to be caused by carboxylate groups, i.e., the bands discussed above at 1596 and 1555 cm^{-1} (^2H_2O) and the band near 1399 cm^{-1}. Others have been assigned to amide I and amide II modes of the protein backbone (see above).

Comparison of phosphorylation spectra obtained with unlabeled ATP and [γ-$^{18}O_3$] ATP

The phosphate region of the spectrum has been investigated in more detail, using caged ATP isotopically labeled at the γ-phosphate ([γ-$^{18}O_3$] caged ATP). Isotopic replacement leads to shifts in the position of phosphate bands, which helps assign the bands in this spectral region. Spectra obtained after release of unlabeled and [γ-$^{18}O_3$] ATP are compared in Fig. 3. As expected, band positions are unchanged by the isotopic replacement above 1300 cm^{-1}, where phosphate groups do not absorb. The agreement of the spectra in that region shows the excellent reproducibility of the small absorbance changes associated with ATPase phosphorylation. Differences are observed below 1300 cm^{-1}, which are due to the isotopic replacement and demonstrate that it is possible to detect the absorbance of the phosphate group in the difference spectra.

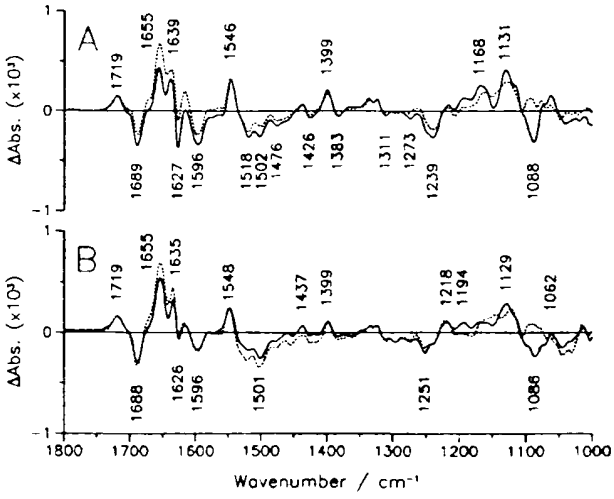

FIGURE 3 Phosphorylation spectrum in H_2O after release of unlabeled ATP (——) and [γ-$^{18}O_3$]ATP (----), pH 7.0, 1°C. (*A*) Type I samples. (*B*) Type II samples.

Only minor isotopic effects are observed in the difference spectrum, the most significant being the decrease in intensity and the alteration of bandshape at 1131 cm^{-1}, as well as the disappearance of the negative band at 1088 cm^{-1}. The position of this negative band, however, does not seem to shift upon isotopic replacement, because it is not observed at a lower wavenumber in the experiments with labeled ATP. Thus its disappearance upon isotopic substitution is most likely due to the superposition of a positive band that is downshifted for the labeled phosphate. The overall effect is to cancel the signals in that spectral region. According to the observed shift of 20–30 cm^{-1} for the $\nu_{as}(PO_3^{2-})$ band of labeled ATP (Takeuchi et al., 1988; Barth et al., 1995), this positive band is expected between 1130 and 1110 cm^{-1}, where indeed the spectra are sensitive to the isotopic replacement. The sensitivity to isotopic substitution of the spectrum near 1131 cm^{-1} and 1088 cm^{-1} supports the tentative assignment (as discussed above) of the 1131 cm^{-1} band to the $\nu_{as}(PO_3^{2-})$ mode of the phosphoenzyme.

Smaller differences not accompanied by an alteration of bandshape are observed between 1300 and 1150 cm^{-1}, some of which are present in only one type of sample. We are therefore careful to attribute these changes to effects of the isotopic replacement. They may also be caused by slight baseline variations, which are also observed above 1300 cm^{-1}.

DISCUSSION

Small changes in the infrared spectrum were observed upon ATP-induced ATPase phosphorylation ($Ca_2E_1 \cdot ATP \rightarrow Ca_2E_1$-P) that were less than 0.2% of total protein absorption. Some of the changes have been tentatively assigned to a conformational change in the protein backbone, and to the absorption of Asp^{351} and the transferred phosphate group (see Results).

Conformational change

The spectra indicate that ATPase phosphorylation is accompanied by a conformational change involving β-sheet, α-helical, and, likely, turn structures (see Results). In the current model of ATPase secondary structure, β-sheets are only predicted in the cytoplasmic domain (Green et al., 1986; MacLennan et al., 1987; MacLennan, 1990). Therefore, the likely location of the conformational change involving β-sheets is the cytoplasmic domain. A conformational change in that part of the protein is supported by the observation of fluorescence changes of fluorescent probes attached to this domain (Suzuki et al., 1994). α-Helices are predicted throughout the protein, and it is thought that α-helices contain the Ca^{2+}-binding ligands located in the membrane part of the protein (Andersen, 1995). It may well be that the small signal of the α-helical segment represents a slight conformational distortion of these helices when the Ca^{2+} ions become occluded.

Ca^{2+}-binding sites

The Ca^{2+}-binding sites are thought to contain several carboxyl groups (Andersen, 1995), and some bands of the phosphorylation spectra can be tentatively assigned to the ν_{as} and ν_s modes of the COO^- group (at 1596 and 1555 cm^{-1} in ^2H_2O, 1430–1380 cm^{-1}). The negative bands in the ν_{as} mode region at 1596 and 1555 cm^{-1} are characteristic for $Ca_2E_1 \cdot ATP$. In principle, bands in that spectral region could originate from the Ca^{2+}-chelating carboxyl groups. Then these bands should appear when the Ca^{2+} complex is formed and thus should be observed as positive bands for the Ca^{2+}-binding reaction $E \rightarrow Ca_2E_1$. However, the Ca^{2+} binding spectra show positive bands at different positions, i.e., at 1606 and 1568 cm^{-1} in H_2O (Georg et al., 1994; Troullier et al., 1996) and ^2H_2O (Georg et al., 1994). Therefore, it is unlikely that the bands in the ν_{as} mode region of the phosphorylation spectra can be attributed to the Ca^{2+} ligands. Instead, one of these bands may be caused by the phosphorylation of Asp^{351} (see Results) or by the several Asp residues that are crucial for phosphorylation (Andersen, 1995).

Whereas no influence of the phosphorylation reaction on the carboxylic Ca^{2+} ligands could be detected, the reaction may affect backbone structure elements that are formed when Ca^{2+} binds to the ATPase. The two minima of the phosphorylation spectrum in the amide I region at 1689 and 1627 cm^{-1} (1680 and 1618 cm^{-1} in ^2H_2O) represent secondary structure elements (see above) of $Ca_2E_1 \cdot ATP$ that are affected by the phosphorylation reaction. The structural element absorbing at 1689 cm^{-1} may have been formed upon Ca^{2+} binding, which is associated with a positive band at nearly the same position. However, most other structural elements of Ca_2E_1 formed upon Ca^{2+} binding do not seem to be affected by the phosphorylation reaction, because most positive bands of the Ca^{2+} binding spectra in the amide I region do not have corresponding negative bands in the phosphorylation spectrum. These positive bands for Ca^{2+} binding were observed in H_2O (^2H_2O) at 1658 (1660), 1642 (1644), and 1632 (1632) cm^{-1} (Buchet et al., 1991a; Georg et al., 1994; Troullier et al., 1996).

In conclusion, most structural features that are affected by Ca^{2+} binding to the ATPase seem to be unaffected by the ATP-induced phosphorylation of the ATPase. This includes details of the secondary structure and the interaction of the Ca^{2+}-chelating carboxylate groups. Instead, most of these characteristics are affected by the subsequent partial reaction of phosphoenzyme conversion and Ca^{2+} release (Barth et al., 1997b).

Interactions of Asp^{351} and phosphate groups

Three bands have been associated with those groups that directly participate in the phosphorylation reaction (see Results): the 1719 cm^{-1} band was tentatively assigned to the C=O group of aspartyl phosphate (Barth et al., 1994, 1996; this work), the 1239 cm^{-1} band to the $\nu_{as}(PO_2^-)$ modes of

the bound ATP, and the 1131 cm^{-1} band has been shown to be caused by a phosphate mode of aspartyl phosphate. The position of the latter band and the position of the C=O band indicate that the phosphate group of the phosphoenzyme is deprotonated: if it were protonated, positive bands would be expected at 1738 cm^{-1} (νC=O), 1237 cm^{-1} (ν_{as} PO_2^-), and 1097 cm^{-1} (ν_s PO_2^-) (see Results), but these are not observed.

The band position of the aspartyl phosphate C=O group is not affected by the isotopic replacement of bulk H_2O by ^2H_2O (Fig. 1, *A* and *B*), or by the addition of 20% Me_2SO (compare Fig. 1, *A* and *B*). In contrast, downshifts of 4 cm^{-1} are observed in both cases for acetyl phosphate. This indicates that the C=O group and/or the phosphate group does not interact with bulk water. In line with this, the bandwidth of the 1719 cm^{-1} band is significantly decreased compared to the bandwidth of acetyl phosphate (see Fig. 2 *C*), which can be explained by a restricted freedom of conformation due to defined interactions of the C=O group with its environment.

The $\nu_{as}(PO_2^-)$ band of the bound ATP near 1234 cm^{-1} seems to be upshifted relative to that of free ATP in the model difference spectrum. In the phosphorylation spectrum, this band is superimposed on its left-hand side, with a positive band due to the side reaction of the photolysis products with glutathione (Barth et al., 1996). If this band is subtracted (not shown) using a control spectrum (Barth et al., 1996) obtained for the same time interval after the release of AMP-PNP, a nonhydrolyzable ATP analog, the minimum of the band appears at 1253 cm^{-1}, i.e., it is shifted by ~20 cm^{-1} with respect to the band of free ATP. If evaluated from the slope at the right-hand side of the band, the shift seems to be somewhat smaller, i.e., 10 cm^{-1}. An upshift of 20–35 cm^{-1} has been observed for the $\nu_{as}(PO_2^-)$ band of several phosphate compounds upon water removal (Brown and Peticolas, 1975; Arrondo et al., 1984; Pohle, 1990; Pohle et al., 1990). Thus the upshift of the $\nu_{as}(PO_2^-)$ band of the bound ATP molecule relative to free ATP in water indicates that the strength of interaction of the α- and/or β-phosphate(s) with the surrounding medium is considerably reduced for the bound ATP, because the phosphate groups are partly dehydrated. Water may be replaced by interactions with a divalent cation (in our case Ca^{2+}) or with protein residues.

Contributions of other amino acid side chains

The lack of the characteristic large isotopic shifts upon protein deuteration for the side chains of Lys, Arg, Asn, and Gln in the 1700–1600 cm^{-1} region of the phosphorylation spectrum make significant contributions of these residues to the spectra unlikely. The infrared spectrum of these residues is expected to be sensitive to a change in the strength of interaction with charged and polar groups. Thus a change in the strength of the interaction between Lys, Arg, Asn, or Gln side chains and their environment was not detected. However, the difference spectra would probably not detect a replacement of one interaction partner by another, which preserves the strength of the interaction. Also, the extinction coefficient of Lys is relatively small (Venyaminov et al., 1990a), and thus Lys absorbance changes may have escaped detection. Mutagenesis studies showed that two Lys residues (Lys^{352} and Lys^{684}) are crucial for ATPase phosphorylation, and Lys^{758} has been suggested to play a role in maintaining the cytoplasmic gate to the Ca^{2+}-binding sites (Andersen, 1995).

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